Anti-CD3 therapy permits regulatory T cells to surmount T cell receptor–specified peripheral niche constraints

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Treatment with anti-CD3 is a promising therapeutic approach for autoimmune diabetes, but its mechanism of action remains unclear. Foxp3$^+$ regulatory T (T reg) cells may be involved, but the evidence has been conflicting. We investigated this issue in mice derived from the NOD model, which were engineered so that T reg populations were perturbed, or could be manipulated by acute ablation or transfer. The data highlighted the involvement of Foxp3$^+$ cells in anti-CD3 action. Rather than a generic influence on all T reg cells, the therapeutic effect seemed to involve an ∼50–60-fold expansion of previously constrained T reg cell populations; this expansion occurred not through conversion from Foxp3$^-$ conventional T (T conv) cells, but from a proliferative expansion. We found that T reg cells are normally constrained by TCR-specific niches in secondary lymphoid organs, and that intraclonal competition restrains their possibility for conversion and expansion in the spleen and lymph nodes, much as niche competition limits their selection in the thymus. The strong perturbations induced by anti-CD3 overcame these niche limitations, in a process dependent on receptors for interleukin-2 (IL-2) and IL-7.

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Abbreviations used: BR, BDC2.5.RAG$^{o/o}$; DT, diphtheria toxin; DTR, DT receptor; γc, common gamma chain; PLN, pancreas-draining lymph node; T conv, conventional T; TCR, T cell receptor; Th, T helper; T reg, regulatory T; TSLP, thymic stromal lymphopoietin.

Treatment with an antibody targeting CD3 is one of the more promising avenues currently being pursued for the therapy of organ-specific autoimmune diseases. Following the precedents from rodent models (Herold et al., 1992; Vallera et al., 1992; Hayward and Shriber, 1992; Chatenoud et al., 1994), administration of anti-CD3 to patients with recently diagnosed diabetes has yielded favorable results in two clinical trials, with a stabilization of disease progression (Herold et al., 2002; Keymeulen et al., 2005). In both mice and humans, anti-CD3 treatment resulted in long-lasting effects that persisted long after clearance of the antibody. However, the mechanism of action is not clear. TCR blockade and internalization, induction of anergy, and perturbation of the T helper (Th) 1/Th2 balance have all been invoked (Hayward and Shriber, 1992; Alegre et al., 1995; Smith et al., 1997). Some studies have suggested an important role for immunosuppression by TGFβ, although conflicting cytokine sources have been proposed (Belghith et al., 2003; Chen et al., 2008; Perruche et al., 2008). More recently,

several investigators have suggested that anti-CD3 therapy may elicit an increase in cells with immunoregulatory properties, in particular Foxp3$^+$ regulatory T (T reg) cells of the CD4$^+$ (You et al., 2007) or CD8$^+$ (Ablamunits and Herold, 2008) lineages.

Foxp3$^+$ T reg cells are the best characterized lymphocyte subset with a regulatory phenotype, playing an important role in the control of antiinfective, antitumor, and autoimmune responses (Belkaid and Rouse, 2005; Roncarolo and Battaglia, 2007; Dougan and Dranoff, 2009). These regulatory activities are manifest via one or more molecular mechanisms (Vignali et al., 2008). The homeostasis of T reg populations is critical to their potency, but is poorly understood. Although cytokines whose receptors use the common γ chain (γc), as well as other molecules, have been shown to

influence the number of peripheral T reg cells, several issues remain unclear: e.g., whether these elements are required purely for peripheral homeostasis or are also involved in thymic differentiation of T reg cells; whether they are involved in proliferation and/or survival; or whether they are implicated only under specific conditions, such as lymphopenia or inflammation.

Some studies on anti–CD3-treated mice have variably shown modifications of T reg cells, sometimes present but quantitatively modest (Belghith et al., 2003; Bresson et al., 2006), sometimes absent (Chen et al., 2008), sometimes restricted to particular anatomical locations (Belghith et al., 2003; Kohm et al., 2005) or involving cells of an unusual CD25^{low} phenotype (You et al., 2007). Certain of the disparate results may have stemmed from the use of CD25 for the identification of T reg cells. This is an issue because NOD mice have an unusually high proportion of the CD25-negative T reg component (Feuerer et al., 2007), which in most other strains constitutes only a minority of Foxp3^{+} cells (Fontenot et al., 2005b).

In this context, we thought it worthwhile to reexamine the impact of anti-CD3 treatment on Foxp3^{+} T reg cells, using some powerful new reagents: mice genetically devoid of T reg cells, mice in which T reg cells can be acutely ablated, and mice in which T reg cell detection is facilitated by fluorescent reporters. The results point in an unexpected direction: anti-CD3 appeared to act by lifting niche limitations on the size (and activity) of particular T reg cell clonotypes, through a striking and selective burst of amplification.

RESULTS
Anti-CD3 treatment prevented diabetes development in BDC2.5.RAG[o/o] mice

There have been conflicting reports on the importance of T reg cells in anti-CD3–induced reversal of diabetes (Belghith et al., 2003; Bresson et al., 2006; Chen et al., 2008). We thus revisited this issue by evaluating the effect of anti-CD3 treatment in two T reg-deficient diabetes-prone strains of mice. Both strains carry the BDC2.5 transgenes (Katz et al., 1993), which encode the two chains of a diabetogenic TCR reactive against a β cell antigen. Diabetes in BDC2.5 mice on the NOD genetic background is highly regulated, such that animals present with a restrained insulitis that seldom progresses to clinical diabetes, except when also harboring mutations leading to a deficiency in CD4^{+}Foxp3^{+} T reg cells (Lühder et al., 1998; Chen et al., 2005). BDC2.5.Foxp3[sf] mice carry the inactivating *scurfy* mutation at the *Foxp3* locus, preventing effective maturation and survival of T reg cells; and BDC2.5.RAG[o/o] (BR) mice are unable to rearrange endogenous TCR genes and appear as a consequence to be devoid of T reg cells. Both of these strains present with very aggressive diabetes, which ensues rapidly after the onset of β cell–derived antigen presentation in the pancreas-draining lymph nodes (PLNs). Mice of the two strains were treated with anti-CD3 or control antibody in the few days around the onset of diabetes (from 12 d of age for BDC2.5.Foxp3[sf] mice and from

17 d of age for BR mice). Diabetes was delayed for a short period in the anti-CD3–treated BDC2.5.Foxp3[sf] mice (Fig. 1 A), but no lasting protection was established, and diabetes appeared essentially in sync with waning of the CD3 blockade on conventional T (T conv) cells (Fig. 1 B). This result established that anti-CD3 could inhibit β-cell destruction for the short term by blocking T conv cells directly, not through T reg cells.

Anti-CD3–treated BR mice were also protected from diabetes development but, in contrast, disease blockade lasted for a longer time in half of them, reminiscent of the protection afforded in recently diagnosed diabetic NOD mice: 50% of anti-CD3–treated BR mice remained normoglycemic to at least 80 d of age (Fig. 1 C). This long-term protection was unexpected because BR mice were thought to be devoid of T reg cells, as are other MHC-II–restricted TCR transgenic mice on a RAG-deficient background (Jordan et al., 2001; Knoechel et al., 2005). To more directly tackle the role of
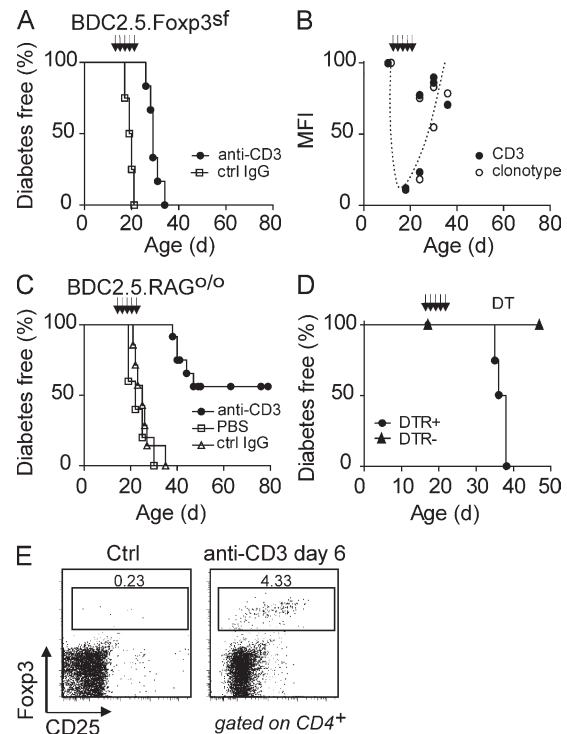

Figure 1.   Anti-CD3 treatment in T reg-deficient diabetic mice.
(A and B) BDC2.5.Foxp3[sf] mice treated with anti-CD3 mAb or control Rat IgG from 12 d of age onwards. (A) Diabetes progression (anti-CD3 mAb, *n* = 6; Rat IgG, *n* = 4). (B) Surface expression of CD3 and BDC2.5 clonotype TCR on CD4^{+} cells from spleens. Two to three mice were analyzed at each time point. (C) Diabetes progression in BR mice treated with anti-CD3 (*n* = 12), PBS (*n* = 5), or control Rat IgG (*n* = 7). (D) Diabetes incidence in BR.Foxp3[DTR+] mice (*n* = 5) or DTR− littermate mice (*n* = 4) treated with anti-CD3 as above. Ablation of T reg cells was provoked by administration of DT at 31, 32, 34, 36, and 38 d of age. Individual arrows indicate each injection of anti-CD3 mAb or control antibody. (E) Splenic CD4^{+} cells from BR mice 6 d after treatment with anti-CD3 or Rat IgG were analyzed by flow cytometry. Numbers indicate the percentage of Foxp3^{+} cells among CD4^{+} cells. The cytograms are representative of four mice per group.
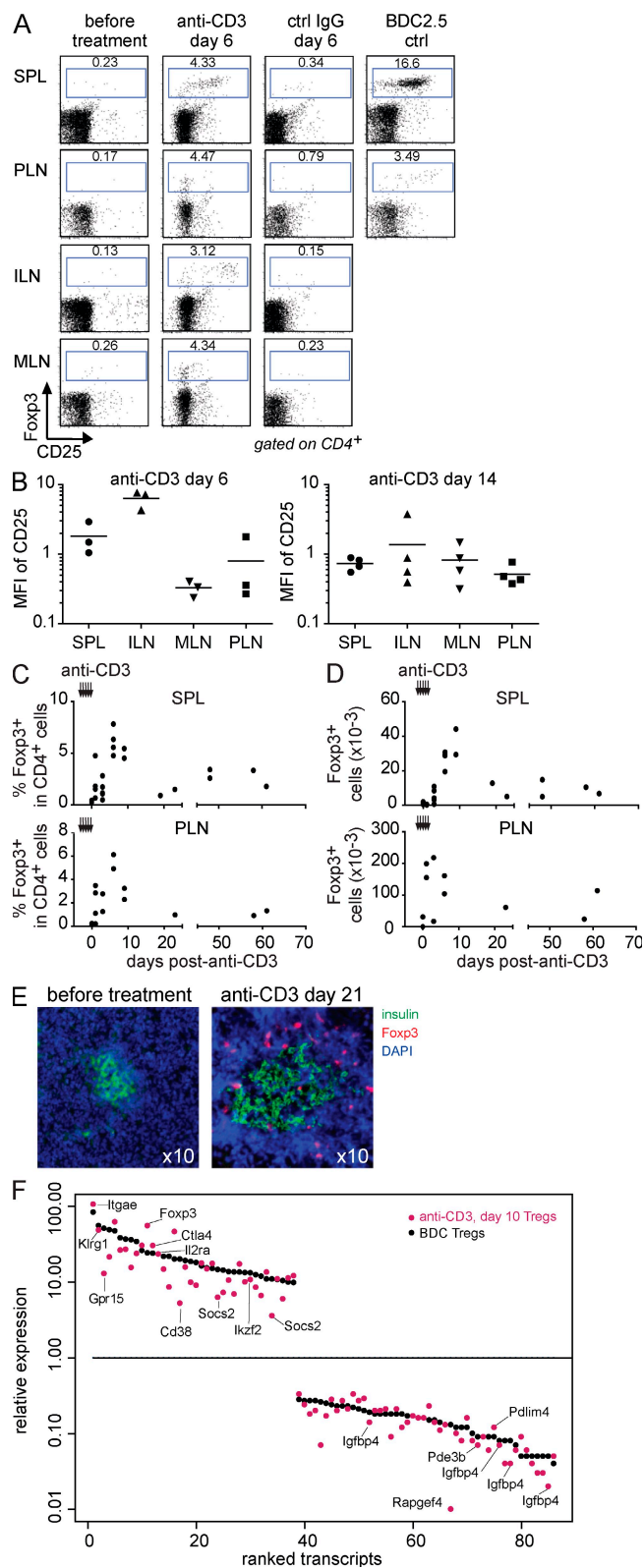
Figure 2. Anti–CD3 induces a burst of T reg cells in BR mice.
(A) CD4+ cells in the spleen, pancreatic, inguinal, and mesenteric lymph node (SPL, PLN, ILN, and MLN, respectively) from mice untreated or treated with anti–CD3 or control IgG were analyzed by flow cytometry.

T reg cells, we took advantage of BDC2.5.Foxp3^{DTR} mice, which express the diphtheria toxin (DT) receptor (DTR) under the control of the Foxp3 promoter, and allow T reg lineage ablation by administration of DT (Feuerer et al., 2009). We generated BR.Foxp3^{DTR} mice (hereafter BR. Foxp3^{DTR}), and young BR.Foxp3^{DTR+} or BR.Foxp3^{DTR-} littermates were treated with anti–CD3, followed by DT. All the anti–CD3–treated BR.Foxp3^{DTR+} mice developed diabetes within 5 d of DT treatment, but their transgene-negative BR.Foxp3^{DTR-} littermates remained protected (Fig. 1 D), confirming that Foxp3+ T reg cells are required for long-term protection. This paradoxical role of Foxp3+ T reg cells, in BR mice from which they were thought absent, prompted us to analyze protected BR mice by flow cytometry. Quite surprisingly, anti–CD3 elicited a very sizeable population of Foxp3+CD25+ T reg cells in BR mice (Fig. 1 E).

A dramatic expansion of T reg cells in anti–CD3–treated BR mice

We next investigated in more detail the kinetics and distribution of the surprising population of Foxp3+ cells elicited by anti–CD3 in BR mice. The appearance of T reg cells was systemic, observed in all secondary lymphoid organs, although the expression of CD25 differed in the spleen and subcutaneous LN, relative to pancreas- and gut-associated LNs (Fig. 2 A). Interestingly, the intensity of CD25 expression initially varied with anatomical location, high in spleen and ILN, lower in MLN or PLN, which one might speculate stems from a different amount of IL-2 in these locations (Fig. 2 B). These varying levels normalized at later times. The Foxp3+ population appeared rapidly upon administration of anti–CD3, with a maximum at day 6–9 after treatment for both their proportion among CD4+ T cells (Fig. 2 C) and their absolute numbers (Fig. 2 D). They waned slightly thereafter, but persisted in the 2–4% range for a long time. Foxp3+ cells were observed in the infiltrated islets of "cured" BR mice, with a scattered disposition reminiscent of that of T reg cells in the restrained insulitis of RAG-positive

CD4+ cells in the SPL and PLN from BDC2.5.NOD mice were depicted as controls. Numbers indicate the percentage of Foxp3+ cells among CD4+ cells. (B) Mean fluorescence intensity of surface CD25 in FoxP3+ cells from different lymphoid organs, 6 or 14 d after anti–CD3 treatment. To normalize for experimental variation between experiments, data were normalized relative to NOD T reg splenocytes used as a staining control in each experiment. (C and D) Percentage and absolute numbers of Foxp3+ CD4+ cells in the SPL and PLN of anti–CD3–treated BR mice. (E) Localization of Foxp3+ cells in residual pancreatic infiltrates from BR mice before or 21 d after treatment with anti–CD3. Data in A–E are representative of three to six independent experiments. (F) Expression of T reg signature transcripts in CD4+Foxp3+ T reg cells elicited by anti–CD3 in cured BR mice, 11 or 12 d after treatment, compared with natural T reg cells from BDC2.5 mice (BDC T regs). RMA-normalized expression values from triplicate Affymetrix M430v2 microarrays were averaged, standardized relative to expression in BDC2.5 T conv cells, and ranked according to differential over- or underexpression in natural BDC2.5 T reg cells.

BDC2.5 mice (Fig. 2 E); in contrast, large infiltrates devoid of Foxp3+ cells were found throughout the pancreas of control BR mice.

The intensity of Foxp3 in these anti-CD3–elicited cells was comparable with those of bona fide T regs (Figs. 1, 2). To further characterize these cells, we determined gene-expression profiles of sorted CD4+GFP+ cells in *Foxp3*^{GFP} knock-in reporter (Fontenot et al., 2005b; R Foxp3^{GFP}) mice, 10 d after anti-CD3 treatment (in triplicate on Affymetrix M430 arrays). These were compared with profiles determined in parallel from normal T reg and T conv cells of BDC2.5.RAG+ mice, used here as a reference for unmanipulated T reg cells. The plot of Fig. 2 F depicts the expression levels (relative to T conv) of transcripts differentially expressed in T reg cells (ranked according to T reg/T conv cell ratios, and focusing on the extremes of the T reg/T conv cell differential). The Foxp3+ T cells induced in anti-CD3–treated BR mice were very similar to T reg cells from BDC2.5 mice, with differential expression of the usual set of diagnostic genes: overexpression of *Foxp3*, *Il2ra*, *Ikzf2*, *Nrp1*, etc., and underexpression of *Pdlim4, Pde3b*, etc. Rare T reg cell signature transcripts stood out as even more differentially expressed in anti-CD3–elicited Foxp3+ cells, relative to BDC2.5 T reg cells (e.g., *Igfbp4, Rapgef,* or even *FoxP3* itself), a few others less so (e.g., *Gpr15, Cd38,* or *Socs2*). We also noted relatively high expression of *Itgae* (CD103) and *Klrg1*, markers of a particular T reg cell subpopulation (Stephens et al., 2007; Beyersdorf et al., 2007; Feuerer et al., 2010). Overall, the cells elicited by anti-CD3 of BR mice seemed like bona fide T reg cells, in terms of anatomical location and genome-wide phenotype.

Origin of T reg cells in anti-CD3–treated BR mice

The sudden appearance of the Foxp3+CD4+ T reg cell population begged the question of its origin. Theoretically, these cells could originate from the thymus, a plausible scenario given the precedent of agonist-driven T reg cell selection (Jordan et al., 2001). Alternatively, they might be of peripheral origin, resulting either from conversion of T conv cells to a Foxp3+ phenotype or from amplification of a preexisting pool of very rare T reg cells (Kretschmer et al., 2005).

Several experiments ruled out a required impact of anti-CD3 in the thymus. First, although Foxp3+ cells did appear in the thymus after anti-CD3 treatment of BR mice (Fig. 3 A), their proportion was comparatively low (only 2% of mature CD4+CD8− thymocytes). In addition, the timing of their induction was largely superimposable to that of peripheral Foxp3+ cells, rendering a precursor–product relationship rather unlikely. More definitively, BR mice thymectomized 1 d before anti-CD3 administration exhibited the characteristic induction of Foxp3+CD4+ cells in peripheral lymphoid organs, which was only marginally less than that of sham-operated BR mice (Fig. 3 B). Thus, the thymus is dispensable during the period of exposure to anti-CD3, and the new population of T reg cells must have a peripheral origin.

Second, we tested the hypothesis that anti-CD3 induces conversion from Foxp3-negative precursors by performing cell transfer experiments using Foxp3− donor cells (Fig. 3 C). The Foxp3^{GFP} knock-in reporter (Fontenot et al., 2005b) was crossed into the BR line, and donor cells were rigorously sorted as GFP− cells to <0.01% contamination, before transfer into age-matched NOD.CD90.1 congenic hosts (a lymphoreplete host was used to avoid the rapid conversion that follows transfer into lymphopenic hosts; Lin et al., 2007; Lathrop et al., 2008; Leung et al., 2009; Feuerer et al., 2010). Hosts were treated the next day with anti-CD3 or control mAb, as above, and tested after 6–8 d. Although some degree of conversion to GFP+ was observed in GFP− cells, it only occurred at a low level and was not different in mice treated with anti-CD3 or control mAb. These results suggest that the
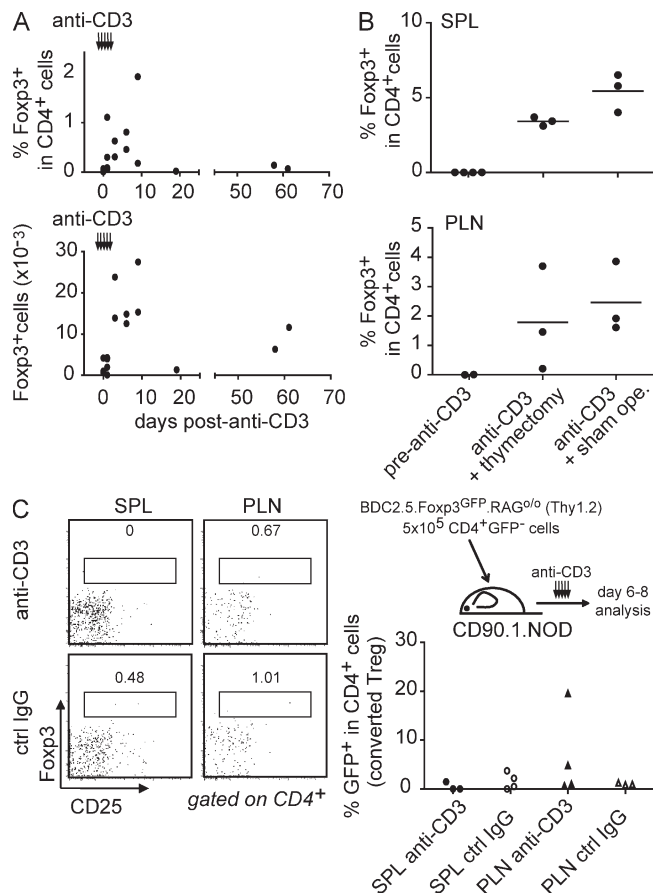

Figure 3. Origin of T reg cells in anti-CD3–treated BR mice.
(A) Percentage and absolute numbers of Foxp3+ CD8−CD4+ cells in the thymus of anti-CD3–treated BR mice. Data are pooled from 3–6 mice per each time point. (B) Percentage of Foxp3+ cells in CD4+ cells in BR mice that were thymectomized or sham-operated before anti-CD3 treatment (n = 3 per each group). (C) GFP− cells from 15-d-old BR.Foxp3^{GFP} mice were transferred into age-matched CD90.1.NOD hosts, anti-CD3 treatment being initiated 24 h after transfer. Six to eight days later, CD4+ T cells from SPL and PLN were analyzed by flow cytometry. Representative flow cytograms are shown at left, and pooled data from four experiments are combined (right).

sudden appearance of the T reg cell population after anti-CD3 treatment of BR mice does not result from conversion of T conv cells. Another caveat was that these Foxp3^{+} cells arise not as a consequence of anti-CD3 itself, but from the large amounts of antigen released at onset of diabetes, which could promote agonist-induced conversion. This caveat was tested by analyzing BR mice maintained after onset of diabetes with insulin pellet; in this case, no increase in T reg cells was observed (Fig. S1).

We then weighed the hypothesis of a rapid amplification of a rare population of T reg cells preexisting in BR mice. Upon close examination of their secondary lymphoid organs, with carefully controlled anti-Foxp3 staining or with BR. Foxp3[GFP] reporter mice, a small but distinct population of T reg cells was detected, representing ∼0.15–0.25% of splenic CD4^{+} T cells (over backgrounds of ∼0.03–0.05%; Fig. 4 A).

These appeared to be true T reg cells, expressing Foxp3 and the usual level of CD25 (Fig. 2). Although this finding was unexpected, as it is usually assumed that TCR transgenic mice on a RAG-deficient background are devoid of T reg cells, it was consistent with very recent studies that very small T reg compartments are selected in the thymus of monoclonal TCR transgenic mice such as BR, but that intraclonal competition between immature thymocytes expressing the same TCR specificity drastically limits the number of selected cells (Bautista et al., 2009; Leung et al., 2009).

Next, we asked whether anti-CD3 might provoke the proliferation of these rare preexisting T reg cells. BR mice received a 6-h pulse of BrdU at different times after anti-CD3 treatment, and incorporation of the label was evaluated by flow cytometry (Fig. 4, B and C). Both T reg and T conv cells proliferated rapidly in the first day after anti-CD3 treatment, but T reg cells continued to expand further, with up to 40% of T reg cells actively cycling a few days after treatment, whereas the expansion of T conv cells subsided quickly after the first day. These differential rates of cell division can certainly account for the expansion of T reg cells from the initial 0.25% to the 5–8% observed 6 d later. The proportion of BrdU–labeled cells suggests
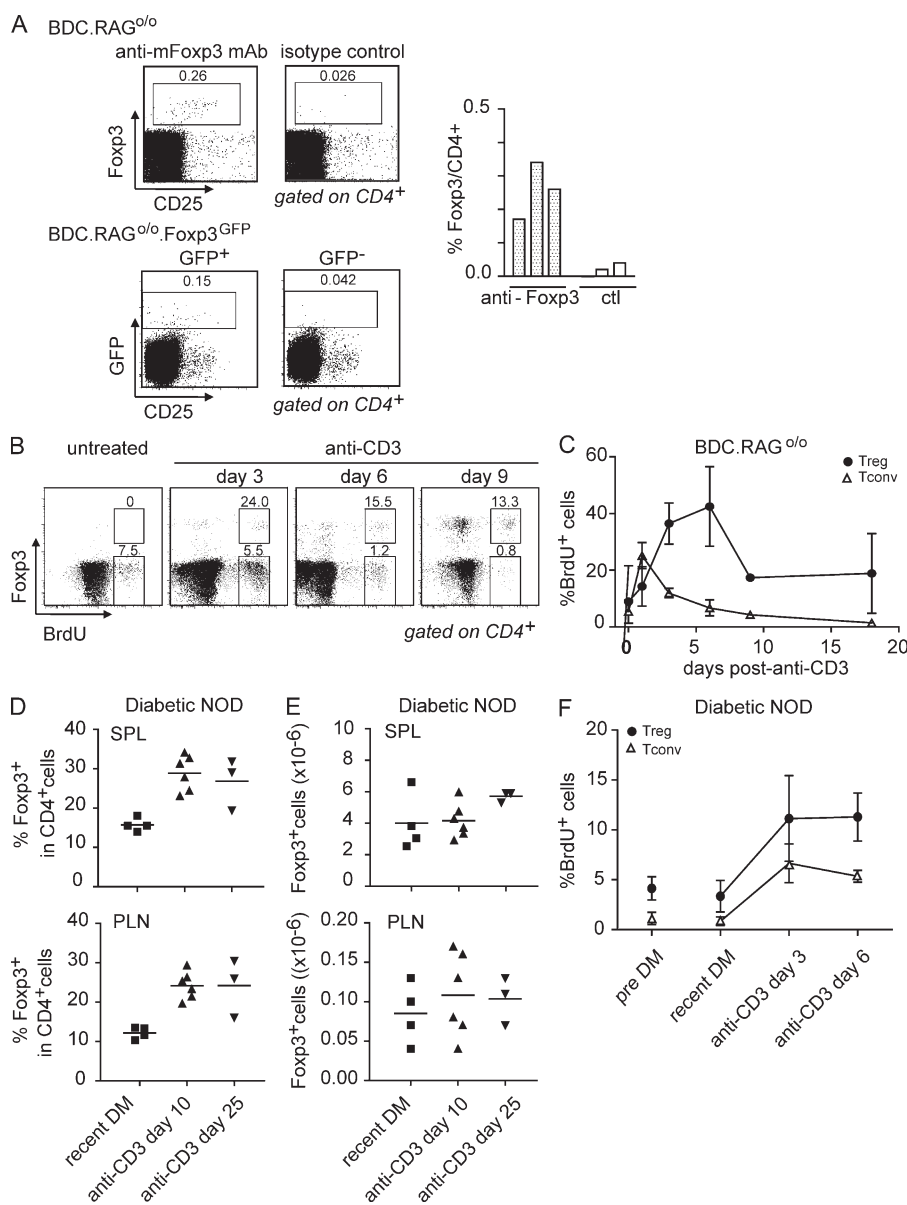

Figure 4. Specific proliferative expansion of T reg cells in anti–CD3–treated BR mice. (A) Anti-Foxp3 or isotype-matched control staining of splenic CD4^{+} cells from an untreated BR mouse at 17 d of age (top); GFP expression in CD4^{+} cells from untreated BR. Foxp3[GFP] mouse (bottom). Note that 10-fold more cells were collected than in previous figures. Numeric data from three mice are shown in the histogram at right. (B) BrdU incorporation by T reg and T conv cell CD4^{+} splenocytes in BR mice during a 6-h pulse, before or at 3, 6, or 9 d of anti-CD3 treatment. The numbers indicate the percentage of BrdU^{+} cells in Foxp3^{+} or Foxp3^{−} CD4^{+} cells. The flow cytograms were representative from three mice per time point. (C) Kinetics of the proportion of BrdU^{+} cells in T reg cells and T conv cells, compiled from several experiments as shown in B. Dots represent mean ± SD of two to five mice per time point. (D and E) Proportion and absolute numbers of CD4^{+}Foxp3^{+} cells in CD4^{+} cells from the spleens and PLNs of NOD mice recently diagnosed as diabetic (recent DM), before or after treatment with anti-CD3. (F) Proportion of BrdU^{+} cells among T reg and T conv SPL cells of NOD mice, in prediabetic state (preDM), recent DM, and 3 or 6 d of anti-CD3 treatment (6-h pulse as above). Dots represent mean ± SD of three mice per time point.
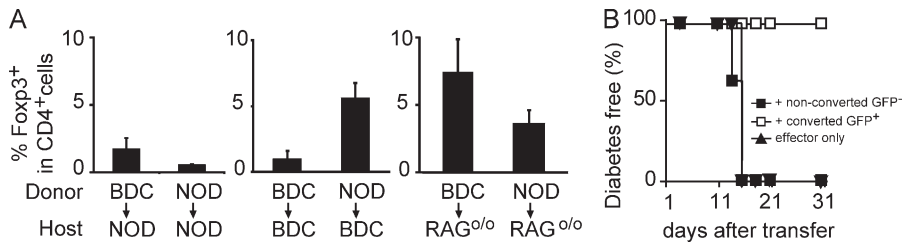
Figure 5. TCR–specific niches in peripheral lymphoid organs. (A) Flow-sorted CD4+GFP− cells from TCR-transgenic BDC2.5+.CD90.1.Foxp3GFP (BDC) or CD90.1.Foxp3GFP (NOD) donors (<0.1% GFP+) were transferred to NOD, BDC2.5, or NOD.RAGo/o hosts, and the proportion of GFP+ cells was measured after 14 d. (B) Diabetes in NOD.RAGo/o recipients of 5 × 10⁴ naive BDC2.5 T cells (effector), alone or with 2.5 × 10⁴ cells recovered from transfer experiments as in A (BDC2.5.CD90.1 either GFP+ converted or GFP− nonconverted). Four mice per group.

a population average division time of ∼10 h, or 8–10 divisions over the 4-d period, potentially leading to a 2^{10} expansion of T reg cell numbers. Thus, anti-CD3 treatment results in a dramatic expansion of these monoclonal T reg cells.

We then asked whether this explosive amplification was particular to the rare T reg cells of BR mice, or whether it was a more general phenomenon. NOD mice recently diagnosed with diabetes were treated with anti-CD3 according to the same regimen. Consistent with previous reports, diabetes reverted in 15 of 37 mice treated (unpublished data). Protected animals showed an increase in the proportions of Foxp3+ T reg cells, though the changes in numbers were less clear (Fig. 4, D and E). In keeping with observations in BR mice, cell cycling was observed in both T reg and T conv, again, preferentially in T reg cells (Fig. 4 F).

TCR–specific niches in peripheral lymphoid organs

Thus, anti-CD3 treatment appeared to unleash the expansion of a monoclonal population of T reg cells, normally selected and maintained at low frequency. Yet, strong homeostatic pressure on T reg cell pools is able to drive the conversion of Foxp3− T conv cells and their expansion to restore peripheral T reg cell pools (Liang et al., 2005; Lin et al., 2007; Feuerer et al., 2010), to a level determined at least in part by dendritic cells (Darrasse-Jèze et al., 2009). If so, why do the rare T reg cells of BR mice (or other such monoclonal TCR mice) not expand to fill the peripheral pools? One hypothesis is that T reg cells of a given specificity can only occupy a niche of restricted size in the periphery.

We tested this hypothesis in a series of transfer experiments, using as donors Foxp3-negative CD4+ cells displaying different TCRs (polyclonal NOD or restricted BDC2.5) identifiable with a CD90.1 congenic marker. These cells were rigorously sorted on the basis of a Foxp3GFP marker, and were transferred into hosts in which T reg cell pools were themselves polyclonal, restricted, or completely missing (NOD, BDC2.5, or NOD.RAGo/o hosts, respectively). There was little, if any, conversion of T conv cells into Foxp3+ T reg cells from polyclonal NOD donors upon transfer into NOD hosts,. This was expected, as transfer of polyclonal cells into lympho-replete hosts is known to elicit little or no conversion. In contrast, the introduction of the same NOD T cells into restricted-repertoire BDC2.5 hosts yielded a robust population of Foxp3+ cells (Fig. 5 A, middle). This behavior did not reflect a generic propensity of BDC2.5 hosts to support T reg conversion and expansion, because transfer of CD4+ T conv cells from BDC2.5.Foxp3GFP mice resulted

in only background levels of GFP+ cells in the same hosts. Both types of donor cells were able to generate Foxp3+ cells upon transfer into an empty NOD.RAGo/o host (Fig. 5, right). These results suggest that the BDC2.5 transgenic hosts, although they do harbor T reg cells, offer several T reg niches for cells displaying TCR specificities different from their own.

The newly generated Foxp3+ cells were very stable, persisting at the same levels for several months, and also remained Foxp3+ through serial retransfer (Fig. S2). They expressed several transcripts typical of the T reg cell signature (unpublished data), and were fully functional, protecting from diabetes in a transfer assay (Fig. 5 B).

If this conversion and expansion of T reg cells were in response to TCR–specific niches left unoccupied in the hosts, one should be able to visualize the specificity and size variability of these niches as an imprint on the expanded repertoire. We have previously shown that T reg and T conv cells in BDC2.5 mice express endogenous TCRα genes, whose products pair with the transgene-encoded TCRβ chain; this diversity allows for selection of a far more robust T reg cell compartment than that of BR mice. Different motifs of rearranged CDR3α segments are used in T reg and T conv cells, which provide a useful "tag" for fate-mapping (Wong et al., 2007). The sequence of TCRα chains expressed by single Foxp3+ cells found 14 d after transfer into NOD.RAGo/o hosts were determined. Fig. 6 A summarizes data from 174 converted Foxp3+ cells, and 165 Foxp3− nonconverted cells, from two independent donor mice (all sequences are tabulated in Table S1). The CDR3α sequences found in these converted and expanded T reg cells were diverse (Fig. 6 A, left). They included sequences normally found in the T conv repertoire (as expected from the conversion event), but also some sequences normally found in T reg cells (perhaps originating from expansion of rare contaminating T reg cells in the donor pool). Importantly, however, the relative distribution of sequences was different from that of either T reg or T conv cells from BDC2.5 mice, indicating that the homeostatic pressure to fill the niches led to a reordering of sequence frequencies. This point is made most explicit in Fig. 6 B, which compares the frequency of CDR3α sequences in T conv cells that became Foxp3+ versus those that remained Foxp3-negative (focusing on sequences of T conv origin). Very different representations were found in the two groups
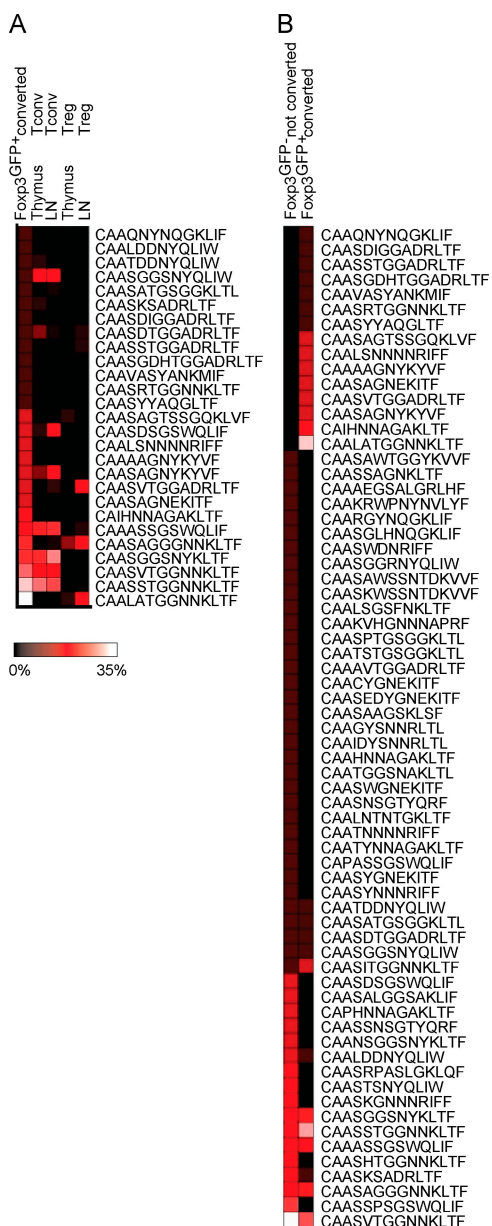
Figure 6. **The TCR repertoire influences the ability to homeostatically convert to Foxp3+ BDC2.5.CD90.1.** Foxp3[GFP+] (converted) and Foxp3[GFP−] (not converted) T cells were isolated 14 d after transfer into lymphopenic hosts as in Fig. 5 A. Vα2+ cells were sorted for single-cell amplification and sequencing of the CDR3α regions of endogenously rearranged TCRα loci (used as a lineage-tracing tag, per Wong et al., 2007). (A) Comparison of the frequencies of CDR3α sequences between Foxp3+ and Foxp3− BDC2.5 T cells, displayed as a frequency heat map. Sequences were aligned based on their frequencies in converted Foxp3+ cells, and compared with the frequencies observed previously in T reg and T conv cells from LN and thymus of BDC2.5 mice (data from Wong et al., 2007). (B) Comparison of the frequencies of CDR3α sequences in transferred cells, either Foxp3+ converted or nonconverted cells that remained Foxp3− (ranked according to frequencies in nonconverted cells). Data were pooled from two independent experiments.

of cells. Similar results were reported by Lathrop et al. (Lathrop et al., 2008) who showed, in a different single–chain TCR system, that homeostatically driven conversion to Foxp3+ phenotype differentially affects cells with different TCRs. Thus, the availability of open niches appears to promote a thorough reordering of TCR frequencies.

Is the size of these niches similar among different organs of the same mouse, and are they influenced by the reactivity of the BDC2.5 clonotype to its pancreatic antigen? After transfer of BDC2.5 T cells into NOD.RAG[o/o] hosts, Foxp3+ cells were found in all secondary lymphoid organs, but most prominently in the subcutaneous lymph nodes and spleen, rather than in the mesenteric or pancreas–draining lymph nodes (Fig. 7, A and B). Similar patterns were found in TCRα[o/o] hosts, indicating that T cell lymphopenia alone was sufficient to drive the conversion and expansion of Foxp3+ cells, irrespective of the B cell pool (Fig. 7 B). A time-course
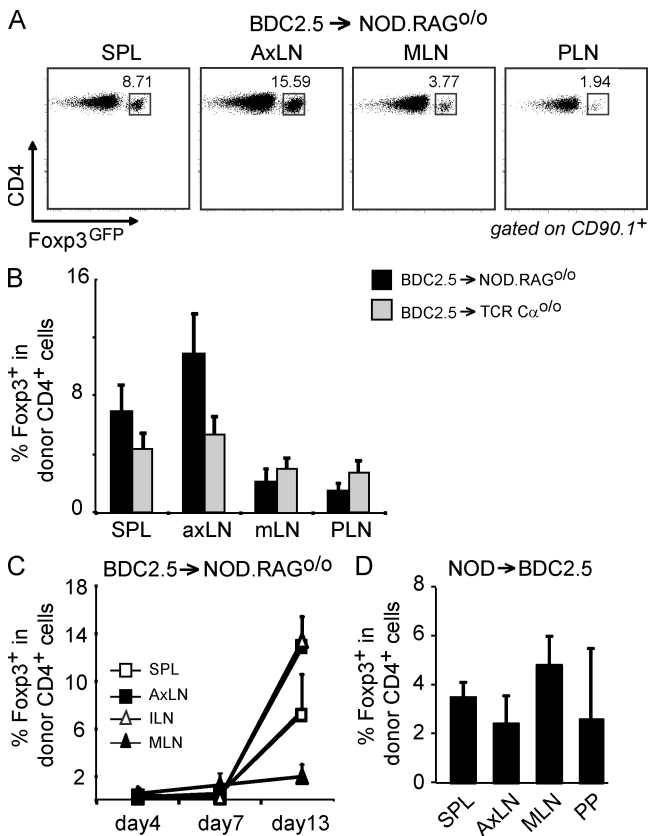

Figure 7. **Anatomical location of TCR–specific T reg niches.** Cells from the SPL, axillary lymph node (AxLN), MLN, and PLN of NOD.RAG[o/o] hosts 14 d after transfer of GFP− cells from BDC2.5.CD90.1.Foxp3[GFP] mice were analyzed by flow cytometry. (A) Representative dot plots. Numbers indicate the percentage of Foxp3+ cells among CD4+ cells. Plots are gated on CD90.1+ T cells. (B) Percentage of Foxp3+ donor cells after transfer of BDC2.5 Foxp3− cells into NOD.RAG[o/o] and TCRCα[o/o] hosts; bars show mean ± SD of four mice per group. (C) Time course of the appearance of Foxp3+ cells after transfer of BDC2.5 Foxp3− cells into NOD.RAG[o/o] hosts. (D) Percentage of Foxp3+ cells after transfer of NOD Foxp3− cells into BDC2.5 hosts; bars show mean ± SD of 4 mice per group.

experiment showed equally low percentages of T reg cells in all the organs at days 4 and 7 after transfer, with a rapid increase in the second week after transfer (Fig. 7 C). An equally broad distribution of Foxp3^{+} cells was also observed after transfer of NOD T conv cells into BDC2.5 hosts (Fig. 7 D). Thus, niche-filling conversion and amplification are not related to the potential autoreactivity of the TCR.

Cytokine involvement in overcoming T reg niche limitations

For T conv cells, homeostatic control is maintained by the existence of TCR-dependent niches, and by limiting trophic cytokines such as IL-7 and IL-15 (Surh and Sprent, 2008; Rochman et al., 2009). We asked which cytokines might be involved in the anti–CD3–mediated surmounting of TCR-specific niche size in BR mice, testing those cytokines known to partake in thymic differentiation of T reg cells and/or their peripheral survival. Co-treatment of antibodies targeting TGFβ (mAb 2G7), along with anti-CD3, showed that this cytokine was not involved, the usual induction of Foxp3^{+} cells being observed in the spleen and PLN (Fig. 8 A). In contrast, coadministration of anti–IL-2 or anti–IL-7R with anti-CD3 strongly reduced the yield of Foxp3^{+} cells, although not quite to the level of controls not receiving anti-CD3 (combination of the two anti–cytokine antibodies had a similar effect; unpublished data). Anti–IL-15, on the other hand, seemed to have no influence. Thus, both IL-2 and IL-7 (or thymic stromal lymphopoietin [TSLP]), which also activates IL-7R) seem to be required for niche resetting. The relevance of IL-2 was confirmed by treating BR mice with IL-2–anti–IL-2 complexes (Boyman et al., 2006), which resulted in a strong expansion of Foxp3^{+} cells (Fig. 8 C).

DISCUSSION

In this study, two quite different lines of experimentation converged to illustrate the strong forces driving T reg cells, through conversion and/or amplification, to fill the restricted niches available to them. These forces are evident after T cell transfer into a host in which the niches are empty (e.g., NOD T cells into BDC2.5 mice; NOD or BDC2.5 T cells into lymphopenic recipients) or when niche limitations are disturbed by extraneous treatment, e.g., administration of anti-CD3. The divergent responses of donor T cell populations expressing different repertoires of TCRs, and the distinct set of TCRα sequences found in converted/amplified T reg cells, both indicate that peripheral homeostasis of the T reg cell pool is set not only at a general population–wide level, but also for individual TCRs or families thereof. In this respect, peripheral T reg niches appear to prolong the intra-clonal competition observed during their selection in the thymus (Bautista et al., 2009; Leung et al., 2009).

Our results suggest that limitations that define T reg cell niches are imposed by both TCRs and receptors for trophic cytokines, acting in combination. The TCR component, manifest as CDR3 sequence specificity, most likely reflects competition for specific MHC–peptide ligands presented by dendritic cells and/or other lymphoid stromal cells. This interpretation is consistent with the recent report that increasing the pool of DCs also increased T reg cell numbers (Darrasse-Jèze et al., 2009). It is possible that these ligands represent a nonrenewable resource, engagement by the TCRs of one T reg cell leading to their internalization, or to stripping of these ligands from the APC surface. Antigen recognition by T cells has long been known to result in uptake of the MHC–peptide complexes by the responding T cell (Lorber et al., 1982; Tsang et al., 2003). Alternatively, the specific MHC–peptide complexes might control the dwell-time allowed to T reg cells on APC surfaces, and competition may be a form of "crowding out" for access to an otherwise stable resource. Whether this TCR-specific limitation represents a strict intra–lineage competition (T reg cells competing among themselves), or an interlineage competition (T conv crowding out T reg cells) remains an open question, and it is
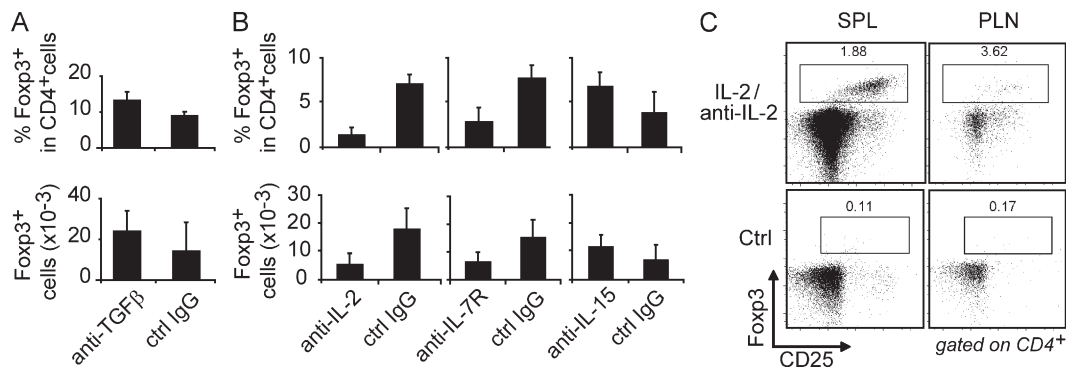

Figure 8. **The appearance of T reg cells after anti–CD3 treatment is dependent on IL–2 and IL–7Ra, but not on TGFβ.** (A) Percentage and absolute numbers of Foxp3^{+} cells among CD4^{+} cells from the spleen of ° BR mice 6 d after treatment with anti-CD3, in combination with anti-TGFβ (2G7) or with control antibody (rat IgG). Antibodies were injected i.p. every other day, starting from the day of anti-CD3 initiation. (B) Percentage and absolute numbers of Foxp3^{+} cells among CD4^{+} cells from the spleen of BR mice 8 d after treatment with anti-CD3 in combination with neutralizing anti-IL-2, anti-IL-7Rα, anti-IL-15, or control IgG. 75 µg of each neutralizing mAb was injected i.p. for 6 consecutive days, starting from the day of anti-CD3 initiation. (A and B) Bars represent mean ± SD of three to four mice per group. (C) IL-2–anti–IL-2 complexes were administered to BR mice for 5 consecutive days from 17 d of age. Cells from SPL and PLN were analyzed by flow cytometry. The cytograms are representative of two to three mice per group.

interesting to note that clonotypic limitations on selection of T reg cells in the thymus have similarly been proposed to reflect T reg/T conv cell (van Santen et al., 2004) or intraclonal T reg/T reg cell competition (Bautista et al., 2009). The cytokine element is represented by IL-2 and IL-7 (or TSLP), consistent with the impact of a deficiency in their receptors on T reg cell homeostasis (Fontenot et al., 2005a; Yao et al., 2006; Bayer et al., 2008). It is not clear from our data whether these cytokines exert complementary effects after anti-CD3 treatment or are merely additive and partially redundant. A limiting supply of cytokines might combine with limiting TCR ligands, for example if a focused delivery of trophic cytokines by the APC is initiated by a productive TCR/MHC–peptide engagement, or if TCR signals sensitize the T reg cell to being receptive to cytokine signals (most simply, by maintaining surface IL-2R or IL-7R levels).

In several respects, the peripheral niche limitations seem to represent a continuation of the intraclonal competition for commitment to the T reg cell lineage observed in the thymus (Bautista et al., 2009; Leung et al., 2009). In particular, the combination of TCR and IL-2R signals is of central importance in T reg cell differentiation and selection in the thymus. As had been speculated for homeostatic control of T conv cells, the peptides that elicit positive selection in the thymus may be the same as those conditioning peripheral survival and niche size.

There are clear parallels between the niche limitations on T reg cells and the homeostatic control of naive T conv cells, whose survival and homeostatically driven proliferation in the periphery are also dependent on continued TCR "tickling" and cytokines (Surh and Sprent, 2008). The niche sizes are different there being far more T conv than T reg cells in monoclonal TCR-transgenic RAG$^{o/o}$ mice, and the required cytokines are different (there being less of a role for IL-2 in T conv cell homeostasis, although IL-7 influences both; Surh and Sprent, 2008; Rochman et al., 2009; Ma et al., 2006). But the operating principles are basically the same. In both cases, setting homeostatic controls linked to individual TCR specificities, rather than operating only at the overall population level, would be an important element to maintain a broad repertoire and avoid takeover by individual clones. Interestingly, though, T reg cells have been shown to influence homeostatic controls on T conv populations (Murakami et al., 2002; Barthlott et al., 2003), and it is also possible that T conv populations play a role in modulating T reg cell homeostasis (although it is easier to imagine generic control rather than TCR-specific niche control).

What happens after anti-CD3 therapy? Perhaps surprisingly, the T reg cells amplified in BR mice seemed quite similar to normal T reg cells from BDC2.5 mice, in transcriptional phenotype. It is plausible that anti-CD3 treatment breaks through the niche ceiling due to a combination of strong TCR- and cytokine-receptor-mediated signals. By analogy, both TCR and cytokine signals are necessary for homeostatic expansion of T conv cells in lymphopenic hosts (Surh and Sprent, 2008; Rochman et al., 2009). Cytokine receptor signals could explain much of the response, given that administration of IL-2/anti–IL-2 complexes largely mimicked the expansion of T reg cells, but the brutal jolt given to T reg cells by anti-CD3 may also promote their expansion. Particularly intriguing was the observation that T reg cells proliferated significantly more than T conv cells in treated BR mice. In polyclonal NOD mice, the same proliferative advantage was observed for T reg cells, albeit numerically more muted; this might suggest that the changes elicited by anti-CD3, which are long-lasting in their protective effect, include a reassortment of the T reg repertoire, with relative amplification of previously limited specificities.

Our findings have several implications for anti-CD3 therapy of autoimmune and other diseases. The KT3 mAb we used, although relatively nontoxic and capable of inducing only a limited "cytokine storm" (Mottram et al., 2002), may have more cytokine-inducing capacity than some of the Fab or non–Fc-binding molecules used in the clinic. Preliminary studies with an F(ab')^2 preparation of the 2C11 mAb injected into BR mice also showed an expansion of the T reg cell pool, but somewhat delayed, peaking at day 14 (unpublished data). It may thus be desirable to test in humanized mouse models the true impact of the preparations used in patients. Our data suggest that a transient therapeutic effect of anti-CD3 treatment might be obtained from the TCR blockade/internalization component, as deduced from the results in BDC2.5.Foxp3^{sf} mice; although short-lived, this effect might be desirable in limiting acute damage in rapidly evolving disease in some patients. A reassortment of the T reg cell repertoires would then ensue, and our data would suggest that the production of IL-2 elicited by these reagents as part of the cytokine storm response, as well as a differential sensitivity, may actually be important contributing elements in the therapeutic effect, via resetting of the T reg niches.

MATERIALS AND METHODS

Mice. BR, BDC2.5.Foxp3^{sf}, Foxp3^{GFP}NOD, and BDC2.5.Foxp3^{DTR} used for intercrosses (all on the NOD/LtJDoi background) have been previously described (Gonzalez et al., 2001; Chen et al., 2005; Feuerer et al., 2009). All were maintained in specific pathogen–free facilities at the Joslin Diabetes Center and Harvard Medical School (IACUC 99–20, 02954).

For anti-CD3 treatment, mice were injected i.v. with 3 µg/g BW of anti-CD3 mAb (clone; KT3; protein G purified) for 5 consecutive days. For T reg depletion in BR.Foxp3^{DTR} mice, 50 µg/g BW of DT was administered at days 1, 2, 4, 6, and 8). For blocking studies, 75 µg of anti–IL-2 (clone; JES6-1A12; BioLegend), anti–IL-7Rα (clone; A7.R34.2.2; protein G purified), and anti–IL-15 (clone; AIO.3; eBioscience) were injected ip for 6 consecutive days, starting on the same day as anti-CD3. 0.5 mg of anti-TGFβ (clone; 2G7; protein G purified) was administered every other day for 2 wk. For in vivo BrdU incorporation, 0.01 mg/g BW of BrdU was injected i.p., 6 h before euthanasia.

Animals were monitored for glycemic control (glycosuria), with confirmation by blood glucose measurements, and mice were considered diabetic if blood glucose levels were >350 mg/dl on two consecutive draws.

Flow Cytometry. Cell surface staining was performed per standard procedures and reagents. Intracellular staining for Foxp3 and BrdU staining was performed with manufacturer's protocols (clone FJK-16s; eBioscience; BrdU Flow kit; BD). For costaining of Foxp3 and BrdU, BrdU staining was performed after Foxp3 staining. Analysis was performed using LSRII instrument (BD) and FlowJo software (Tree Star, Inc.).

Immunofluorescence. Frozen sections of pancreas were fixed with acetone, blocked with 5% FCS, and stained with guinea pig anti–bovine insulin serum (Linco Research) and Alexa Fluor 546–conjugated anti-CD4 mAb (clone; RM4-5; BD) and FITC-conjugated anti–guinea pig IgG antibodies. After a 2-min incubation with 0.1% Triton X-100 and subsequent blocking with 5% FCS, the sections were stained with Alexa Fluor 647–conjugated anti-Foxp3 mAb (clone; FJK-16s; eBioscience), and the nuclei was counterstained with DAPI.

Adoptive T cell transfer. CD4$^+$CD8α$^-$B220$^-$ T cells were sorted by flow cytometry as strictly Foxp3-GFP negative from spleen and LNs of BR.Foxp3^{GFP} mice, BDC2.5.CD90.1.Foxp3^{GFP} mice, or CD90.1.Foxp3^{GFP}. 5×10^5 cells from BR mice were transferred to CD90.1.NOD hosts, which were treated with anti-CD3 from the next day onward as described in Fig. 3. 10^5 cells from BDC2.5 and NOD mice were transferred, and mice were analyzed at different time points.

In vivo T reg cell protection assay. Foxp3^{GFP+} and Foxp3^{GFP-} CD4 T cells were isolated 14 d after transfer of Foxp3^{GFP-}CD90.1$^+$ BDC2.5 T cells into NOD.RAG$^{o/o}$ hosts. Converted GFP$^+$ and GFP$^-$ (not converted) CD4$^+$ T cells were mixed with naive BDC2.5 T cells (effector cell) at a ratio of 1:2 and transferred into NOD.RAG$^{o/o}$ hosts.

Single cell sorting, RT–PCR, and Vα2 sequence analysis. Experiments were performed as previously described (Wong et al., 2007). In brief, BDC2.5 T cells were sorted 14 d after transfer of flow-sorted Foxp3^{GFP-} CD4 T cells into NOD.RAG$^{o/o}$ hosts, as Vα2$^+$BDCclonotype$^+$CD4$^+$CD8α$^-$B220$^-$ and either GFP$^+$ or GFP$^-$, (converted and nonconverted cells, respectively), first in bulk then resorted as individual cells into wells of 96-well PCR plates containing 12 µl complete RT reaction mix. The plates were incubated for 90 min at 37°C, and then heat inactivated for 10 min at 70°C. Plates were replicated by transferring 5 µl of the cDNA into an empty plate. Nested PCR amplification was performed for Foxp3 or Vα2 as previously described (Wong et al., 2007), with strict controls for contamination (1/3 of the wells served as no-cells controls). Vα2 amplifications were prepared for automated sequencing by digestion with Shrimp Alkaline Phosphatase (GE Healthcare) and Exonuclease I (New England Biolabs), and the products sequenced by automated Sanger sequencing (Dana-Farber/Harvard Cancer Center High-Throughput Sequencing Core). Raw sequencing files were filtered for sequence quality, processed for recognition of V, J, and CDR3 elements with a custom PERL script, and the data for each cell were tabulated in an MS-Access database, together with the cell's origin, surface phenotype, and Foxp3 RT-PCR result.

Microarray analysis. CD4$^+$GFP$^+$ splenocytes were sorted from BR Foxp3^{GFP} mice 11–12 d after antiCD3 treatment, as were CD4$^+$GFP$^+$ T reg or CD4$^+$GFP$^-$ T conv splenocytes from BDC2.5 mice, collected directly into Trizol reagent. RNA was amplified for two rounds using the MessageAmp aRNA kit (Ambion), followed by biotin labeling using the BioArray High Yield RNA Transcription Labeling kit (Enzo Diagnostics). The resulting cRNAs were hybridized to M430 2.0 chips (Affymetrix). Raw .cel files were background-corrected and RMA-normalized, and replicates were averaged. Microarray data available from the National Center for Biotechnology Information/GEO repository under accession no. GSE22527.

Online supplemental material. Fig. S1 shows Foxp3$^+$ cells arise not as a consequence of large amounts of antigen released at onset diabetes. Fig. S2 shows converted Foxp3$^+$ cells in NOD.RAG$^{o/o}$ host is stable. Table S1 shows all CDR3α sequences of the converted Foxp3$^+$ or nonconverted Foxp3$^-$ cells found in each host mouse.Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20100205/DC1.

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